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Thematic Review Series: Glycosylphosphatidylinositol (GPI) Anchors:  
Biochemistry and Cell Biology

# GPI-AP release in cellular, developmental, and reproductive biology

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**Abstract** Glycosylphosphatidylinositol-anchored proteins (GPI-APs) contain a covalently linked GPI anchor located on outer cell membranes. GPI-APs are ubiquitously conserved from protozoa to vertebrates and are critical for physiological events such as development, immunity, and neurogenesis in vertebrates. Both membrane-anchored and soluble GPI-APs play a role in regulating their protein conformation and functional properties. Several pathways mediate the release of GPI-APs from the plasma membrane by vesiculation or cleavage. Phospholipases and putative substrate-specific GPI-AP-releasing enzymes, such as NOTUM, glycerophosphodiesterase 2, and angiotensin-converting enzyme, have been characterized in mammals. Here, the protein modifications resulting from the cleavage of the GPI anchor are discussed in the context of its physiological functions.—Fujihara, Y., and M. Ikawa. GPI-AP release in cellular, developmental, and reproductive biology. *J. Lipid Res.* 2016. 57: 538–545.

**Supplementary key words** genetics • gene expression • membranes • phospholipases • phospholipids/phosphatidylinositol • glypican • GPIase • IZUMO1 receptor/JUNO • reversion-inducing cysteine-rich protein with kazal motifs • testis-expressed gene 101 • glycosylphosphatidylinositol-anchored protein

Eukaryotic organisms produce many membrane proteins that are anchored to the outer cell membrane by glycosylphosphatidylinositol (GPI). GPI-anchored proteins (GPI-APs) have unique structures that contain mannose, glucosamine, ethanolamine, and phosphatidylinositol (1). Although they have similar core backbone structures, GPI-APs are a functionally diverse family of molecules that includes hydrolytic enzymes, adhesion molecules, receptors, protease inhibitors, and complement regulatory proteins (2). GPI-APs are released from the cell surface by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC)

(3). In mammals, GPI-APs are released and taken up by both vesiculation-mediated and non-vesiculation-mediated mechanisms (4). Several putative substrate-specific enzymes release GPI-APs from the cell surface (5–8). Thus, GPI-anchor release may be required for different biological functions and regulatory mechanisms. In this review, we will focus on the significance of GPI-AP release in cellular, developmental, and reproductive biology (Table 1).

## GPI-ANCHOR-RELEASING ENZYME, GPLD1/GPI-PLD, TARGETED FOR VARIOUS SUBSTRATES

A unique feature of GPI-APs is their cleavage by specific phospholipases, resulting in the release of the protein from the cell membrane. Among GPI-specific phospholipases, the only purified and characterized mammalian phospholipase is a GPI-specific phospholipase D (GPI-PLD), GPLD1, which is abundant in serum (9–11). GPLD1 is a soluble protein with two functional domains, an N-terminal catalytic domain and a predicted C-terminal  $\beta$ -propeller domain (12, 13). GPLD1 hydrolyzes GPI anchors that have acylated inositol, unlike GPI anchors cleaved by PI-PLC and GPI-PLC

Abbreviations: ACE, angiotensin-converting enzyme; ADAM3, a disintegrin and metallopeptidase domain 3; CEA, carcinoembryonic antigen; CEACAM, carcinoembryonic antigen-related cell-adhesion molecule; DALLY, division abnormally delayed; DLL1, Delta-like 1; DLP, division abnormally delayed-like protein; GDE, glycerophosphodiesterase; GDEP, glycerophosphodiester phosphodiesterase; GPC, glypican; GPI, glycosylphosphatidylinositol; GPI-AP, glycosylphosphatidylinositol-anchored protein; GPIase, glycosylphosphatidylinositol-anchored protein-releasing; GPI-PLC, glycosylphosphatidylinositol-specific phospholipase C; GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D; IZUMO1R, IZUMO1 receptor; LY6K, lymphocyte antigen 6 complex locus K; PGAP1, post-GPI attachment to proteins 1; PI-PLC, phosphatidylinositol-specific phospholipase C; PLAUR, plasminogen activator urokinase; PLAUR, PLAUR receptor; PRSS, protease serine; RECK, reversion-inducing cysteine-rich protein with kazal motifs; SPAM1, sperm-adhesion molecule 1; TDGF1, teratocarcinoma-derived growth factor 1; TEX101, testis-expressed gene 101; ZP, zona pellucida.

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TABLE 1. GPI-APs and GPIase factors involved in cleavage of the GPI anchor from the cell surface

GPI-APs	GPI-AP Releasing Factors	Domain Responsible for GPIase Activity	Phenotype of GPI-AP KO Mice	References
PLUAR/uPAR	GPLD1/GPI-PLD	Catalytic domain	Mild phenotype	(18, 19)
CEACAM5/CEA	GPLD1/GPI-PLD	Catalytic domain	Not determined <sup>a</sup>	(25)
PRSS8/prostasin	GPLD1/GPI-PLD	Catalytic domain	Embryonic lethality	(29, 31)
TDGF1/CRIPTO-1	GPLD1/GPI-PLD	Catalytic domain	Embryonic lethality	(37, 38)
GPC3 (glypicans)	NOTUM	Not determined <sup>a</sup>	Postnatal lethality	(44, 58)
RECK	GDPD5/GDE2	GDPD domain	Embryonic lethality	(5, 67)
TEX101	ACE	Not determined <sup>a</sup>	Male infertility	(6, 7)
IZUMO1R/JUNO	Not determined <sup>a</sup>	Not determined <sup>a</sup>	Female infertility	(104)

<sup>a</sup>Has not been reported.

(3). The catalytic activity of mouse GPLD1 depends on histidines at positions 29, 125, 133, and 158 in the catalytic site (14). Membrane-bound GPI-AP is released from the cell surface by GPLD1 in several important cellular processes, including adhesion, differentiation, proliferation, survival, and oncogenesis. Although the physiological role of GPLD1 is unknown, GPLD1 substrates have been identified, and their roles have been investigated in gene KO mice (Table 1).

#### PLASMINOGEN ACTIVATOR UROKINASE RECEPTOR

Plasminogen activator urokinase receptor (PLAUR, also known as CD87 and uPAR) is a GPI-AP receptor that binds to plasminogen activator urokinase (PLAU, also known as uPA) (15). PLUAR was identified as a multifunctional cell-surface receptor that regulates cellular differentiation, proliferation, migration, adhesion, and invasion (15, 16). When bound to a PLAU, PLAUR functions as a signaling receptor that converts plasminogen into plasmin, which upon activation triggers an extracellular proteolysis cascade (17). PLAUR also interacts nonproteolytically with vitronectin, an interaction that induces the migration of various cell types. PLAUR activity is regulated by receptor shedding and cleavage. Receptor shedding is mediated by cleavage close to the GPI anchor by GPLD1 and several proteases (18), whereas receptor cleavages release the N-terminal ligand binding domain (D1) from the other domains (D2D3) (15). These modifications are independent, and the resulting forms of PLAUR have different biological activities. Although minor phenotypes were observed in *Pluar* KO mice, PLUAR was dispensable for development and fertility in mice (19).

#### CARCINOEMBRYONIC ANTIGEN AND RELATED ADHESION MOLECULES

Carcinoembryonic antigen (CEA), first described in 1965, is a highly glycosylated immunoglobulin-related protein whose serum level increases in some types of cancer (20, 21). CEA-related cell-adhesion molecules (CEACAMs) have been implicated in numerous physiological and pathological functions (22). In humans, there are three major CEACAMs on epithelial tissues: one transmembrane type (CEACAM1) and two GPI-anchored types (CEACAM5 and CEACAM6) (23). Because *Ceacam1* KO mice have no gross abnormalities, epithelial CEACAMs seem to contribute to the fine-tuning of

cellular events (24). CEACAMs may be more important under pathological conditions, as they are bound by various pathogens (22). CEACAM5 is released by GPLD1-mediated GPI-anchor cleavage (25); it is overexpressed in many cancers and is a widely used tumor biomarker (20, 21). The analysis of *Ceacam5* KO mice has not been reported yet.

#### PRSS8/PROSTASIN

Protease serine (PRSS)8 (also known as prostasin) is a trypsin-like serine peptidase encoded by the PRSS8 gene. It was purified as a soluble enzyme from human seminal fluid and is highly expressed in kidney, lung, pancreas, and prostate in mice and humans (26, 27). As a GPI-AP, PRSS8 can be removed from the cell membrane by treatment with PI-PLC (28). Under physiological conditions, PRSS8 secretion from the membrane-bound form depends on GPI-anchor cleavage by endogenous GPLD1 in epithelial cells (29). PRSS8 and the type II transmembrane serine protease, matriptase/ST14, are generally recognized to interact with serine protease inhibitors (SPINT1 and SPINT2) and are indispensable for epithelial development and homeostasis (30). PRSS8 is required for terminal epidermal differentiation and postnatal survival in mice (31), but its enzymatic activity is not required for development and postnatal homeostasis (32). Thus, the nonenzymatic activity of PRSS8 is essential for in vivo development. The role of GPLD1-mediated release of PRSS8 in vivo has not been determined.

#### TERATOCARCINOMA-DERIVED GROWTH FACTOR 1

Teratocarcinoma-derived growth factor 1 (TDGF1) is a GPI-AP and a member of the epidermal growth factor-CFC (CRIPTO-1/FRL-1/cryptic) family. TDGF1 interacts with various components of multiple signal pathways to enhance stem cell renewal, epithelial-mesenchymal transition, proliferation, and oncogenesis (33). TDGF1 acts as both a ligand and coreceptor in the nodal signaling pathway that involves Smad 2, 3, and 4 (34, 35). GPI-anchored TDGF1 is required for paracrine activity as a nodal coreceptor (36). In its soluble form, TDGF1 functions as a ligand in tumor progression. Because the shedding of TDGF1 is mediated by GPLD1 activity at the GPI-anchor site, soluble TDGF1 may stimulate endothelial cell migration and tumor angiogenesis (37). TDGF1 is essential for

embryogenesis and cellular transformation in mice (38), but the physiological functions regulated by the membrane-bound and soluble forms have not been determined (34).

### NOTUM-MEDIATED GPI-ANCHOR CLEAVAGE IN DEVELOPMENT

Glypicans (GPCs) are heparin sulfate proteoglycans that are bound to the cell surface of the plasma membrane by a GPI anchor (39). Two GPCs, division abnormally delayed (DALLY) and Dally-like protein (DLP), have been identified in *Drosophila* (40, 41). The six GPC family proteins (GPC1–6) have been conserved in mice and humans (39, 42–44). GPCs regulate signaling pathways triggered by Hedgehogs, Wnts, bone morphogenetic proteins, and fibroblast growth factors, and function in axon guidance and excitatory synapses (45). GPI-anchored GPCs interact directly with Wnt family proteins and can stimulate Wnt signaling by facilitating or stabilizing the interaction between Wnts and their receptors (46–50). GPCs may also inhibit Wnt signaling (47). Moreover, in *Drosophila*, secreted GPCs have been reported to play a role in the transport of Wnts in the imaginal wing discs for regulation of morphogen gradient formation during development (51–54).

NOTUM, a member of the  $\alpha/\beta$ -hydrolase superfamily, regulates Wnt activity by modifying the heparin sulfate chains of GPCs, DALLY, and DLP (55). NOTUM was reported to induce shedding of DLP from the cell surface by cleaving its GPI anchor (8) (Table 1). However, the idea that NOTUM mediates release of GPCs was called into question by a report that recombinant *Drosophila* NOTUM cannot cleave DLP and instead functions as a carboxylesterase that removes an essential palmitoleate moiety from Wnt proteins (56). *Xenopus* NOTUM is also a Wnt deacylase and is required for brain development (57). Mammalian NOTUM was reported to cleave not only GPCs (GPC3, GPC5, and GPC6), but also other GPI-APs (PLAUR and T-cadherin) (58). Further study is needed to resolve questions about the function of NOTUM and its enzymatic activities.

### GPI-ANCHOR CLEAVAGE OF RECK BY GLYCEROPHOSPHODIESTERASE 2 IN NEUROGENESIS

Glycerophosphodiester phosphodiesterases (GDPDs) are periplasmic and cytosolic proteins that are critical for

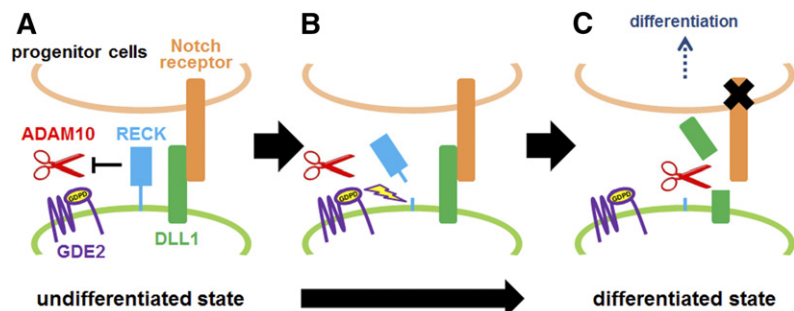
the hydrolysis of deacylated glycerophospholipids to glycerol phosphate and alcohol, which are then utilized as a major source of carbon and phosphate (59). Though GDPDs are highly conserved from bacteria to mammals, they differ in their enzymatic activities and biological functions. The mammalian glycerophosphodiesterases (GDEs) have substrate specificities that do not need glycerophosphodiester. Seven homologs (GDE1–7) are conserved in mice and humans. The physiological roles of the GDE family were recently reviewed (60).

GDE2 (also known as GDPD5) has six transmembrane domains and an extracellular GDPD domain (61). GDE2 is an osmoregulated enzyme (62) and a key regulator of motor neuron differentiation (63). GDE2 is expressed in undifferentiated progenitors, but not in mature motor neurons, and its GDPD activity induces non-cell-autonomous motor neuron generation by inhibiting Notch signaling (64). Though the mechanism used by GDE2 to inhibit Notch signaling cannot be explained by conventional GDPD activity, the GPI-AP-releasing (GPIase) activity of GDE2 promotes this process during neural differentiation (5).

Reversion-inducing cysteine-rich protein with kazal motifs (RECK) is a GPI-AP that activates Notch signaling in cortical progenitors by directly inhibiting ADAM10, a disintegrin, and metalloproteinase 10, which removes the Notch ligand Delta-like 1 (DLL1) (65–67). GDE2's GPIase activity is derived from the GDPD activity domain, which sheds the GPI anchor of RECK in a manner different from that of GPI-PLD (5) (Table 1). Thus, GDE2-mediated RECK release controls the timing of DLL1-Notch signal-mediated neurogenesis (Fig. 1). GDE3 and GDE6 may have similar GPIase activities (59). Their physiological functions remain to be determined in vivo.

### ROLE OF GPI-APS IN FERTILIZATION

Fertilization is a complicated multi-step event. In mammals, sperm ejaculated into the female reproductive tract must migrate through the utero-tubal junction to the ampulla, where fertilization takes place. Oocytes released from the ovary are surrounded by an extracellular matrix, the zona pellucida (ZP), and cumulus cell layers filled with hyaluronic acid. At the ampulla, spermatozoa must cross the cumulus cell layer, penetrate the ZP, interact with the oocyte membrane, and finally fuse with the egg. Experiments in KO mice have been undertaken to classify the various factors that are essential for fertilization (68, 69).



**Fig. 1.** GDE2 GDPD activity-dependent cleavage of RECK during neurogenesis. A: RECK inhibits ADAM10-mediated DLL1 cleavage (65, 66). DLL1-Notch signaling maintains the undifferentiated state of neuronal progenitor cells. B: GDE2 with its GPIase activity residing in a GDPD domain releases RECK from the cell surface (5). C: The release of RECK enables ADAM10 to access and cleave DLL1 (107). The subsequent discontinuation of Notch signaling non-cell-autonomously induces neural differentiation of progenitor cells.



GPI-APs and their removal play key roles during fertilization. Below, we will discuss the current understanding of the functions of GPI-APs in mammalian fertilization.

### GPI-APs and sperm migration in the female reproductive tract

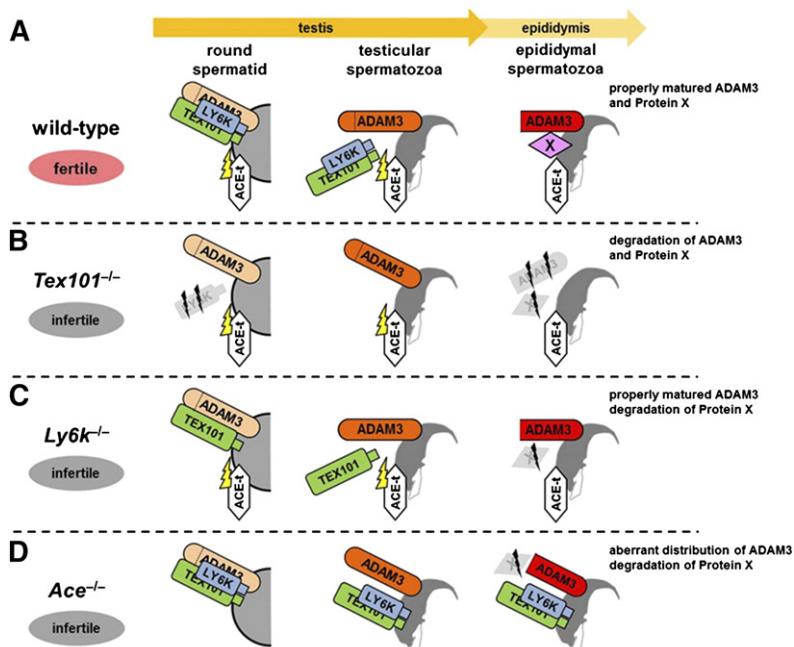
GPI-APs on the surface of spermatozoa originate primarily from de novo synthesis by spermatogenic cells and by transfer from epididymal luminal fluids, likely mediated by exosomes (70). Three surface-bound GPI-APs, CD52, CD55, and CD59, are thought to protect spermatozoa from immunological attack in the female reproductive tract (71). Removal of these GPI-APs was thought to regulate sperm capacitation, which readies sperm for the acrosome reaction (72). However, studies in KO mice showed that the fertilization ability of sperm does not require CD52, CD55, CD59a, and CD59b (73–76). In spermatogenesis, CD59b has a critical role that is independent from complement regulatory function (74). Recently, *Cd59b* was identified in mice, but its physiological function remains to be determined.

During the migration of sperm through the female reproductive tract, the utero-tubal junction is a critical barrier. GPI-APs are directly or indirectly involved in this step. Post-GPI attachment to proteins 1 (PGAP1) is GPI inositol-deacylase that resides in the endoplasmic reticulum and contributes to the maturation of GPI-APs (77). *Pgap1* KO mice have otocephaly and growth retardation, and often die right after birth. Males that survive to sexual maturity are severely subfertile, probably because they have three “footed” GPI anchors that are resistant to cleavage of sperm GPI-APs (78). The mutant spermatozoa are impaired in their ability to migrate from the uterus into the oviduct in vivo and bind to the ZP in vitro. The same phenotype has been reported in KO mice lacking a disintegrin and metalloproteinase domain 3 (ADAM3), a cysteine-rich glycosylated

sperm membrane protein (79, 80). However, the link between GPI-APs and ADAM3 has not been elucidated.

More than 10 molecules have been reported to be indispensable for ADAM3 maturation and the fertilizing ability of spermatozoa (68). Male mice lacking angiotensin-converting enzyme (ACE), a zinc peptidase that regulates blood pressure (81), were subfertile and had low blood pressure because of the lack of somatic and testicular ACE isoforms (82, 83). *Ace* KO spermatozoa were deficient in their ability to migrate into the oviduct and bind ZP in vitro, leading to the aberrant distribution of ADAM3 (84). Using sperm-adhesion molecule 1 (SPAM1/PH20) and PRSS21 (PRSS21/TESP5) as substrates, ACE also has GPIase activity in spermatozoa (7); however, the GPIase activity was mild and was not confirmed by other groups (85, 86).

Testis-expressed gene 101 (TEX101) and the TEX101-interacting protein, lymphocyte antigen 6 complex locus K (LY6K), are germ-cell-specific GPI-APs found in the testis, but not on epididymal spermatozoa (87, 88). The GPI-AP complex consisting of TEX101 and LY6K interacts with testicular ADAM3 and is required for sperm-fertilizing ability in mice (6, 89). ADAM3 is absent from the Triton X-114 detergent-enriched phase of *Ace* KO spermatozoa (84). These findings raised the possibility that removal of the TEX101/LY6K complex is mediated by ACE's GPIase activity, which could reasonably explain the link between GPI-APs and ADAM3. In vitro analysis in a HEK293T culture system showed that TEX101, not LY6K, is the specific substrate for ACE and that dipeptidase-defective ACE retains its GPIase activity (6, 7, 89) (Table 1). These findings are consistent with the aberrant persistence of the TEX101/LY6K complex on spermatozoa in *Ace* KO mice. Thus, ACE's GPIase activity in the formation of fertile spermatozoa is independent of its well-known dipeptidase activity (Fig. 2).



**Fig. 2.** Fate of GPI-AP complex, TEX101/LY6K, ADAM3, and ACE during spermatogenesis. **A:** In round spermatids, a GPI-AP complex consisting of TEX101 and LY6K and an immature form of ADAM3 were both localized on the plasma membrane (6, 89). The association with TEX101 is essential for ADAM3 (pale orange to dark orange) to be spliced properly to form mature ADAM3 (red) in spermatozoa. **B:** Without TEX101, LY6K disappeared from testicular germ cells. ADAM3 (gray) on epididymal spermatozoa was degraded during epididymal maturation, and TEX101 KO male mice were infertile (6). **C:** Without LY6K, TEX101 and ADAM3 seemed to be processed normally during spermatogenesis. LY6K KO male mice were sterile, even though ADAM3 localized normally in LY6K KO spermatozoa (89). This implies the existence of a key factor (Protein X) in mammalian spermatozoa other than ADAM3. **D:** The removal of the TEX101/LY6K complex was mediated by ACE. Without ACE, TEX101/LY6K remained on spermatozoa, and the localization of ADAM3 on epididymal spermatozoa became aberrant. ACE KO mice became infertile, possibly because of nonfunctional ADAM3 (6, 84).

### GPI-APs and sperm-oocyte extracellular matrix interaction

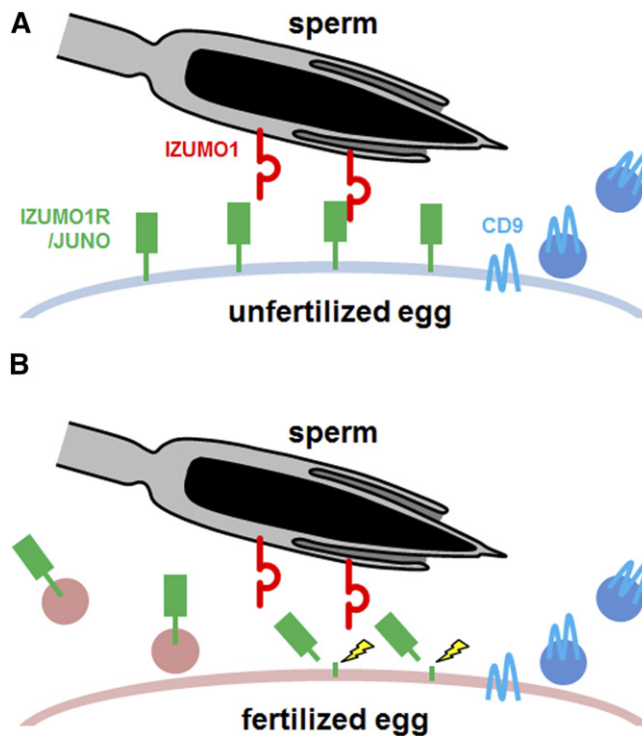
When spermatozoa arrive in the oviduct, they encounter ovulated eggs surrounded by a layer of cumulus cells. SPAM1 was identified as a sperm GPI-anchored receptor for the ZP in guinea pigs and was later reported to have hyaluronidase activity that enables spermatozoa to pass through the cumulus-cell oocyte complex in mice (90–92). *Spam1* KO mice are fertile, and *Spam1*-deficient spermatozoa bind to the ZP, although they have a reduced ability to disperse cumulus cells via hyaluronidase activity in vitro (93). PRSS21, another surface GPI-AP, helps spermatozoa penetrate the ZP (94). However, *Prss21* KO mice are fertile, and their spermatozoa can penetrate the ZP despite the absence of PRSS21 (95). While they may play a role in fertilization, these GPI-APs did not affect in vivo fertilization in the KO experiments.

### GPI-APs and sperm-egg fusion

Sperm-egg fusion is one of the last steps of fertilization. After acrosome reaction and ZP penetration, spermatozoa fuse with egg. Until recently, only two factors were known to be essential for fusion: the transmembrane protein CD9 in the egg and IZUMO1 (named after a Japanese shrine dedicated to marriage) in spermatozoa (96–99). However, recombinant IZUMO1 can bind to *Cd9* KO oocytes, suggesting the existence of IZUMO1-specific ligands rather than CD9 on the egg surface (100).

Besides these transmembrane proteins, there is evidence that GPI-APs on the egg plasma membrane have an essential role in fusion. When mouse oocytes are treated with PI-PLC, GPI-AP clusters are released from the egg surface and spermatozoa fail to fuse with the eggs (101). In addition, oocyte-specific depletion of GPI-AP phosphatidylinositol glycan class A, which is essential for GPI-AP synthesis (102), resulted in female infertility due to an impaired ability to fuse with spermatozoa (103). The GPI-AP responsible for fusion was not known until 2014, when Gavin J. Wright's group, using pentamerized IZUMO1 as bait, identified the GPI-anchored folate receptor 4 as the IZUMO1 receptor (IZUMO1R), which was named JUNO after the Roman goddess of fertility and marriage (104) (Table 1). They also showed that *Izumo1r* KO females are infertile and that *Izumo1r*-deficient eggs cannot fuse with spermatozoa. The interaction between IZUMO1 in spermatozoa and IZUMO1R in eggs is essential for sperm-egg fusion during fertilization (Fig. 3A).

Fertilized oocytes need to prevent fusion with other spermatozoa. Although highly expressed on the surface of unfertilized eggs, IZUMO1R is undetectable on the egg membrane within 40 min after fertilization. IZUMO1R is redistributed into vesicles, presumably derived from the microvillus-rich oolemma, within the perivitelline space (104). This postfertilization disappearance of IZUMO1R prevents polyspermy. These findings demonstrate the importance of IZUMO1R for sperm-egg fusion and shed light on the novel mechanism and physiological function of the removal of GPI-APs from the egg surface (Fig. 3B).




**Fig. 3.** Molecular mechanism of sperm-egg fusion. A: IZUMO1 and CD9 have been identified as essential factors for sperm-egg fusion (96–99). IZUMO1 is an acrosomal membrane protein that is exposed after the acrosome reaction. Fusion-competent spermatozoa exhibit the equatorial segment pattern of IZUMO1 in acrosome-reacted spermatozoa (108). Except at the metaphase plate, CD9 is localized on the cilia, distributed across the surface of unfertilized eggs. CD9-containing vesicles released from the egg surface interact with the sperm surface (109) and may enhance sperm-egg fusion. However, interaction between the two factors has not been observed. Recently, the egg binding partner for IZUMO1 on the egg surface, IZUMO1R/JUNO, was identified as a GPI-AP (104). B: After fertilization, IZUMO1R is rapidly shed from the egg membrane through an unknown mechanism, possibly mediated by vesiculation or by an unidentified GPIase activity. These events may be a possible mechanism for the polyspermy block at the plasma membrane in eggs.

### CONCLUDING REMARKS

Cell-surface GPI-APs are released by GPIase activity in many important biological events, including cellular proliferation, development, neurogenesis, and reproduction. However, it has been challenging to identify the responsible enzymes and mechanisms because GPI-APs can be released from the membrane by many mechanisms, such as vesiculation and cleavages at the peptide or GPI-anchor domains. In this review, we focused on the well-characterized GPIases (GPI-PLD, NOTUM, GDE2, and ACE), but some questions remain to be addressed. Although other groups have questioned the GPIase activity of ACE, we recently showed that ACE has substrate-specific GPIase activity that is independent of its dipeptidase activity. The catalytic domains and the cleaving mechanism involved need to be clarified. On the other hand, surprisingly, NOTUM acts as an extracellular protein deacylase rather than a GPIase (56, 57). Further experiments are required

to establish that NOTUM does not have any GPIase activity.

Most of the GPIases and their substrates have been identified and analyzed in vitro, and their physiological functions have not been investigated in vivo. In vivo studies will be facilitated by using the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 system, a genome-manipulation technology that will enable us to generate gene-disrupted mice within a few months (105, 106). With this system, enzymes and substrates can be tagged, so their fates can be traced and the proteins they interact with in vivo can be identified. In addition, point mutations can be introduced to identify the catalytic domains and substrate recognition sites.

The release of GPI-APs is also important from the clinical point of view. Like CEACAM5 and TDGF1, some circulating GPI-APs can be used as clinical biomarkers of disease (22, 33). Because GPIase has substrate specificity, it could also be a potential target for drug development. Given their location and function in the extracellular space, GPI-APs are also good therapeutic targets. Small-molecule screening will help decipher the mechanism of release of GPI-APs and will also open new avenues for the development of drugs to treat GPI-associated diseases. 

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## REFERENCES

- Ferguson, M. A. J., T. Kinoshita, and G. W. Hart. 2009. Glycosylphosphatidylinositol anchors. In *Essentials of Glycobiology*. A. Varki, R. D. Cummings, J. D. Esko, et al., editors. Cold Spring Harbor Press, Cold Spring Harbor, NY.
- Kinoshita, T. 2014. Biosynthesis and deficiencies of glycosylphosphatidylinositol. *Proc. Jpn. Acad., Ser. B, Phys. Biol. Sci.* **90**: 130–143.
- Ikezawa, H. 2002. Glycosylphosphatidylinositol (GPI)-anchored proteins. *Biol. Pharm. Bull.* **25**: 409–417.
- Tsai, Y. H., X. Liu, and P. H. Seeberger. 2012. Chemical biology of glycosylphosphatidylinositol anchors. *Angew. Chem. Int. Ed. Engl.* **51**: 11438–11456.
- Park, S., C. Lee, P. Sabharwal, M. Zhang, C. L. Meyers, and S. Sockanathan. 2013. GDE2 promotes neurogenesis by glycosylphosphatidylinositol-anchor cleavage of RECK. *Science*. **339**: 324–328.
- Fujihara, Y., K. Tokuihara, Y. Muro, G. Kondoh, Y. Araki, M. Ikawa, and M. Okabe. 2013. Expression of TEX101, regulated by ACE, is essential for the production of fertile mouse spermatozoa. *Proc. Natl. Acad. Sci. USA*. **110**: 8111–8116.
- Kondoh, G., H. Tojo, Y. Nakatani, N. Komazawa, C. Murata, K. Yamagata, Y. Maeda, T. Kinoshita, M. Okabe, R. Taguchi, et al. 2005. Angiotensin-converting enzyme is a GPI-anchored protein releasing factor crucial for fertilization. *Nat. Med.* **11**: 160–166.
- Kreuger, J., L. Perez, A. J. Giraldez, and S. M. Cohen. 2004. Opposing activities of Dally-like glypican at high and low levels of Wingless morphogen activity. *Dev. Cell*. **7**: 503–512.
- Metz, C. N., G. Brunner, N. H. Choi-Muira, H. Nguyen, J. Gabilove, I. W. Caras, N. Altszuler, D. B. Rifkin, E. L. Wilson, and M. A. Davitz. 1994. Release of GPI-anchored membrane proteins by a cell-associated GPI-specific phospholipase D. *EMBO J.* **13**: 1741–1751.
- Low, M. G., and A. R. Prasad. 1988. A phospholipase D specific for the phosphatidylinositol anchor of cell-surface proteins is abundant in plasma. *Proc. Natl. Acad. Sci. USA*. **85**: 980–984.
- Davitz, M. A., D. Hereld, S. Shak, J. Krakow, P. T. Englund, and V. Nussenzweig. 1987. A glycan-phosphatidylinositol-specific phospholipase D in human serum. *Science*. **238**: 81–84.
- Heller, M., P. Butikofer, and U. Brodbeck. 1994. Generation by limited proteolysis of a catalytically active 39-kDa protein from the 115-kDa form of phosphatidylinositol-glycan-specific phospholipase D from bovine serum. *Eur. J. Biochem.* **224**: 823–833.
- Springer, T. A. 1997. Folding of the N-terminal, ligand-binding region of integrin alpha-subunits into a beta-propeller domain. *Proc. Natl. Acad. Sci. USA*. **94**: 65–72.
- Raikwar, N. S., R. F. Bowen, and M. A. Deeg. 2005. Mutating His29, His125, His133 or His158 abolishes glycosylphosphatidylinositol-specific phospholipase D catalytic activity. *Biochem. J.* **391**: 285–289.
- Blasi, F., and P. Carmeliet. 2002. uPAR: a versatile signalling orchestrator. *Nat. Rev. Mol. Cell Biol.* **3**: 932–943.
- Ragno, P. 2006. The urokinase receptor: a ligand or a receptor? Story of a sociable molecule. *Cell. Mol. Life Sci.* **63**: 1028–1037.
- Blasi, F., and N. Sidenius. 2010. The urokinase receptor: focused cell surface proteolysis, cell adhesion and signaling. *FEBS Lett.* **584**: 1923–1930.
- Wilhelm, O. G., S. Wilhelm, G. M. Escott, V. Lutz, V. Magdolen, M. Schmitt, D. B. Rifkin, E. L. Wilson, H. Graeff, and G. Brunner. 1999. Cellular glycosylphosphatidylinositol-specific phospholipase D regulates urokinase receptor shedding and cell surface expression. *J. Cell. Physiol.* **180**: 225–235.
- Bugge, T. H., T. T. Suh, M. J. Flick, C. C. Daugherty, J. Romer, H. Solberg, V. Ellis, K. Dano, and J. L. Degen. 1995. The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. *J. Biol. Chem.* **270**: 16886–16894.
- Goldenberg, D. M., R. M. Sharkey, and F. J. Primus. 1976. Carcinoembryonic antigen in histopathology: immunoperoxidase staining of conventional tissue sections. *J. Natl. Cancer Inst.* **57**: 11–22.
- Gold, P., and S. O. Freedman. 1965. Specific carcinoembryonic antigens of the human digestive system. *J. Exp. Med.* **122**: 467–481.
- Tchoupa, A. K., T. Schuhmacher, and C. R. Hauck. 2014. Signaling by epithelial members of the CEACAM family - mucosal docking sites for pathogenic bacteria. *Cell Commun. Signal.* **12**: 27.
- Kuespert, K., S. Pils, and C. R. Hauck. 2006. CEACAMs: their role in physiology and pathophysiology. *Curr. Opin. Cell Biol.* **18**: 565–571.
- Leung, N., C. Turbide, M. Olson, V. Marcus, S. Jothy, and N. Beauchemin. 2006. Deletion of the carcinoembryonic antigen-related cell adhesion molecule 1 (Ceacam1) gene contributes to colon tumor progression in a murine model of carcinogenesis. *Oncogene*. **25**: 5527–5536.
- Yamamoto, Y., E. Hirakawa, S. Mori, Y. Hamada, N. Kawaguchi, and N. Matsura. 2005. Cleavage of carcinoembryonic antigen induces metastatic potential in colorectal carcinoma. *Biochem. Biophys. Res. Commun.* **333**: 223–229.
- Verghese, G. M., Z. Y. Tong, V. Bhagwandin, and G. H. Caughey. 2004. Mouse prostasin gene structure, promoter analysis, and restricted expression in lung and kidney. *Am. J. Respir. Cell Mol. Biol.* **30**: 519–529.
- Yu, J. X., L. Chao, and J. Chao. 1994. Prostatin is a novel human serine proteinase from seminal fluid. Purification, tissue distribution, and localization in prostate gland. *J. Biol. Chem.* **269**: 18843–18848.
- Chen, L. M., M. L. Skinner, S. W. Kauffman, J. Chao, L. Chao, C. D. Thaler, and K. X. Chai. 2001. Prostatin is a glycosylphosphatidylinositol-anchored active serine protease. *J. Biol. Chem.* **276**: 21434–21442.
- Verghese, G. M., M. F. Gutknecht, and G. H. Caughey. 2006. Prostatin regulates epithelial monolayer function: cell-specific Gpld1-mediated secretion and functional role for GPI anchor. *Am. J. Physiol. Cell Physiol.* **291**: C1258–C1270.
- Szabo, R., and T. H. Bugge. 2011. Membrane-anchored serine proteases in vertebrate cell and developmental biology. *Annu. Rev. Cell Dev. Biol.* **27**: 213–235.
- Hummeler, E., A. Dousse, A. Rieder, J. C. Stehle, I. Rubera, M. C. Osterheld, F. Beermann, S. Frateschi, and R. P. Charles. 2013. The channel-activating protease CAP1/Prss8 is required for placental labyrinth maturation. *PLoS One*. **8**: e55796.
- Peters, D. E., R. Szabo, S. Friis, N. A. Shylo, K. Uzzun Sales, K. Holmbeck, and T. H. Bugge. 2014. The membrane-anchored serine protease prostatin (CAP1/PRSS8) supports epidermal development and postnatal homeostasis independent of its enzymatic activity. *J. Biol. Chem.* **289**: 14740–14749.



33. Klauzinska, M., N. P. Castro, M. C. Rangel, B. T. Spike, P. C. Gray, D. Bertolette, F. Cuttitta, and D. Salomon. 2014. The multifaceted role of the embryonic gene Cripto-1 in cancer, stem cells and epithelial-mesenchymal transition. *Semin. Cancer Biol.* **29**: 51–58.
34. Yan, Y. T., J. J. Liu, Y. Luo, C. E. R. S. Haltiwanger, C. Abate-Shen, and M. M. Shen. 2002. Dual roles of Cripto as a ligand and coreceptor in the nodal signaling pathway. *Mol. Cell. Biol.* **22**: 4439–4449.
35. Whitman, M. 2001. Nodal signaling in early vertebrate embryos: themes and variations. *Dev. Cell.* **1**: 605–617.
36. Watanabe, K., S. Hamada, C. Bianco, M. Mancino, T. Nagaoka, M. Gonzales, V. Bailly, L. Strizzi, and D. S. Salomon. 2007. Requirement of glycosylphosphatidylinositol anchor of Cripto-1 for trans activity as a Nodal co-receptor. *J. Biol. Chem.* **282**: 35772–35786.
37. Watanabe, K., C. Bianco, L. Strizzi, S. Hamada, M. Mancino, V. Bailly, W. Mo, D. Wen, K. Miatkowski, M. Gonzales, et al. 2007. Growth factor induction of Cripto-1 shedding by glycosylphosphatidylinositol-phospholipase D and enhancement of endothelial cell migration. *J. Biol. Chem.* **282**: 31643–31655.
38. Ding, J., L. Yang, Y. T. Yan, A. Chen, N. Desai, A. Wynshaw-Boris, and M. M. Shen. 1998. Cripto is required for correct orientation of the anterior-posterior axis in the mouse embryo. *Nature.* **395**: 702–707.
39. Filmus, J., M. Capurro, and J. Rast. 2008. Glypicans. *Genome Biol.* **9**: 224.
40. Baeg, G. H., X. Lin, N. Khare, S. Baumgartner, and N. Perrimon. 2001. Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. *Development.* **128**: 87–94.
41. Nakato, H., T. A. Futch, and S. B. Selleck. 1995. The division abnormally delayed (dally) gene: a putative integral membrane proteoglycan required for cell division patterning during post-embryonic development of the nervous system in Drosophila. *Development.* **121**: 3687–3702.
42. Jen, Y. H., M. Musacchio, and A. D. Lander. 2009. Glypican-1 controls brain size through regulation of fibroblast growth factor signaling in early neurogenesis. *Neural Dev.* **4**: 33.
43. Aikawa, T., C. A. Whipple, M. E. Lopez, J. Gunn, A. Young, A. D. Lander, and M. Korc. 2008. Glypican-1 modulates the angiogenic and metastatic potential of human and mouse cancer cells. *J. Clin. Invest.* **118**: 89–99.
44. Cano-Gauci, D. F., H. H. Song, H. Yang, C. McKerlie, B. Choo, W. Shi, R. Pullano, T. D. Piscione, S. Grisaru, S. Soon, et al. 1999. Glypican-3-deficient mice exhibit developmental overgrowth and some of the abnormalities typical of Simpson-Golabi-Behmle syndrome. *J. Cell Biol.* **146**: 255–264.
45. Filmus, J., and M. Capurro. 2014. The role of glypicans in Hedgehog signaling. *Matrix Biol.* **35**: 248–252.
46. Song, H. H., W. Shi, Y. Y. Xiang, and J. Filmus. 2005. The loss of glypican-3 induces alterations in Wnt signaling. *J. Biol. Chem.* **280**: 2116–2125.
47. Capurro, M. I., Y. Y. Xiang, C. Lobe, and J. Filmus. 2005. Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. *Cancer Res.* **65**: 6245–6254.
48. Ohkawara, B., T. S. Yamamoto, M. Tada, and N. Ueno. 2003. Role of glypican 4 in the regulation of convergent extension movements during gastrulation in *Xenopus laevis*. *Development.* **130**: 2129–2138.
49. De Cat, B., S. Y. Muyldermans, C. Coomans, G. Degeest, B. Vanderschueren, J. Creemers, F. Biemar, B. Peers, and G. David. 2003. Processing by proprotein convertases is required for glypican-3 modulation of cell survival, Wnt signaling, and gastrulation movements. *J. Cell Biol.* **163**: 625–635.
50. Ai, X., A. T. Do, O. Lozynska, M. Kusche-Gullberg, U. Lindahl, and C. P. Emerson, Jr. 2003. QSOX1 remodels the 6-O sulfation states of cell surface heparan sulfate proteoglycans to promote Wnt signaling. *J. Cell Biol.* **162**: 341–351.
51. Yan, D., and X. Lin. 2009. Shaping morphogen gradients by proteoglycans. *Cold Spring Harb. Perspect. Biol.* **1**: a002493.
52. Han, C., D. Yan, T. Y. Belenkaya, and X. Lin. 2005. Drosophila glypicans Dally and Dally-like shape the extracellular Wingless morphogen gradient in the wing disc. *Development.* **132**: 667–679.
53. Franch-Marro, X., O. Marchand, E. Piddini, S. Ricardo, C. Alexandre, and J. P. Vincent. 2005. Glypicans shunt the Wingless signal between local signalling and further transport. *Development.* **132**: 659–666.
54. Kirkpatrick, C. A., B. D. Dimitroff, J. M. Rawson, and S. B. Selleck. 2004. Spatial regulation of Wingless morphogen distribution and signaling by Dally-like protein. *Dev. Cell.* **7**: 513–523.
55. Giráldez, A. J., R. R. Copley, and S. M. Cohen. 2002. HSPG modification by the secreted enzyme Notum shapes the Wingless morphogen gradient. *Dev. Cell.* **2**: 667–676.
56. Kakugawa, S., P. F. Langton, M. Zebisch, S. A. Howell, T. H. Chang, Y. Liu, T. Feizi, G. Bineva, N. O'Reilly, A. P. Snijders, et al. 2015. Notum deacylates Wnt proteins to suppress signalling activity. *Nature.* **519**: 187–192.
57. Zhang, X., S. M. Cheong, N. G. Amado, A. H. Reis, B. T. MacDonald, M. Zebisch, E. Y. Jones, J. G. Abreu, and X. He. 2015. Notum is required for neural and head induction via Wnt deacylation, oxidation, and inactivation. *Dev. Cell.* **32**: 719–730.
58. Traister, A., W. Shi, and J. Filmus. 2008. Mammalian Notum induces the release of glypicans and other GPI-anchored proteins from the cell surface. *Biochem. J.* **410**: 503–511.
59. Yanaka, N. 2007. Mammalian glycerophosphodiester phosphodiesterases. *Biosci. Biotechnol. Biochem.* **71**: 1811–1818.
60. Corda, D., M. G. Mosca, N. Ohshima, L. Grauso, N. Yanaka, and S. Mariggio. 2014. The emerging physiological roles of the glycerophosphodiesterase family. *FEBS J.* **281**: 998–1016.
61. Nogusa, Y., Y. Fujioka, R. Komatsu, N. Kato, and N. Yanaka. 2004. Isolation and characterization of two serpentine membrane proteins containing glycerophosphodiester phosphodiesterase, GDE2 and GDE6. *Gene.* **337**: 173–179.
62. Gallazzini, M., J. D. Ferraris, and M. B. Burg. 2008. GDE2 is a glycerophosphocholine phosphodiesterase that osmotically regulates the osmoprotective organic osmolyte GPC. *Proc. Natl. Acad. Sci. USA.* **105**: 11026–11031.
63. Rao, M., and S. Sockanathan. 2005. Transmembrane protein GDE2 induces motor neuron differentiation in vivo. *Science.* **309**: 2212–2215.
64. Sabharwal, P., C. Lee, S. Park, M. Rao, and S. Sockanathan. 2011. GDE2 regulates subtype-specific motor neuron generation through inhibition of Notch signaling. *Neuron.* **71**: 1058–1070.
65. Muraguchi, T., Y. Takegami, T. Ohtsuka, S. Kitajima, E. P. Chandana, A. Omura, T. Miki, R. Takahashi, N. Matsumoto, A. Ludwig, et al. 2007. RECK modulates Notch signaling during cortical neurogenesis by regulating ADAM10 activity. *Nat. Neurosci.* **10**: 838–845.
66. Rhee, J. S., and L. M. Coussens. 2002. RECKing MMP function: implications for cancer development. *Trends Cell Biol.* **12**: 209–211.
67. Oh, J., R. Takahashi, S. Kondo, A. Mizoguchi, E. Adachi, R. M. Sasahara, S. Nishimura, Y. Imamura, H. Kitayama, D. B. Alexander, et al. 2001. The membrane-anchored MMP inhibitor RECK is a key regulator of extracellular matrix integrity and angiogenesis. *Cell.* **107**: 789–800.
68. Okabe, M. 2015. Mechanisms of fertilization elucidated by gene-manipulated animals. *Asian J. Androl.* **17**: 646–652.
69. Ikawa, M., N. Inoue, A. M. Benham, and M. Okabe. 2010. Fertilization: a sperm's journey to and interaction with the oocyte. *J. Clin. Invest.* **120**: 984–994.
70. Sullivan, R., G. Frenette, and J. Girouard. 2007. Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. *Asian J. Androl.* **9**: 483–491.
71. Kirchhoff, C., and G. Hale. 1996. Cell-to-cell transfer of glycosylphosphatidylinositol-anchored membrane proteins during sperm maturation. *Mol. Hum. Reprod.* **2**: 177–184.
72. Boerke, A., J. van der Lit, F. Lolicato, T. A. Stout, J. B. Helms, and B. M. Gadella. 2014. Removal of GPI-anchored membrane proteins causes clustering of lipid microdomains in the apical head area of porcine sperm. *Theriogenology.* **81**: 613–624.
73. Yamaguchi, R., K. Yamagata, H. Hasuwa, E. Inano, M. Ikawa, and M. Okabe. 2008. Cd52, known as a major maturation-associated sperm membrane antigen secreted from the epididymis, is not required for fertilization in the mouse. *Genes Cells.* **13**: 851–861.
74. Qin, X., N. Krumrei, L. Grubisich, M. Dobarro, H. Aktas, G. Perez, and J. A. Halperin. 2003. Deficiency of the mouse complement regulatory protein Mcd59b results in spontaneous hemolytic anemia with platelet activation and progressive male infertility. *Immunity.* **18**: 217–227.
75. Holt, D. S., M. Botto, A. E. Bygrave, S. M. Hanna, M. J. Walport, and B. P. Morgan. 2001. Targeted deletion of the CD59 gene causes spontaneous intravascular hemolysis and hemoglobinuria. *Blood.* **98**: 442–449.



76. Sun, X., C. D. Funk, C. Deng, A. Sahu, J. D. Lambiris, and W. C. Song. 1999. Role of decay-accelerating factor in regulating complement activation on the erythrocyte surface as revealed by gene targeting. *Proc. Natl. Acad. Sci. USA*. **96**: 628–633.
77. Tanaka, S., Y. Maeda, Y. Tashima, and T. Kinoshita. 2004. Inositol deacylation of glycosylphosphatidylinositol-anchored proteins is mediated by mammalian PGAP1 and yeast Bst1p. *J. Biol. Chem.* **279**: 14256–14263.
78. Ueda, Y., R. Yamaguchi, M. Ikawa, M. Okabe, E. Morii, Y. Maeda, and T. Kinoshita. 2007. PGAP1 knock-out mice show otocephaly and male infertility. *J. Biol. Chem.* **282**: 30373–30380.
79. Shamsadin, R., I. M. Adham, K. Nayernia, U. A. Heinlein, H. Oberwinkler, and W. Engel. 1999. Male mice deficient for germ-cell cyritestin are infertile. *Biol. Reprod.* **61**: 1445–1451.
80. Yamaguchi, R., Y. Muro, A. Isotani, K. Tokuhito, K. Takumi, I. Adham, M. Ikawa, and M. Okabe. 2009. Disruption of ADAM3 impairs the migration of sperm into oviduct in mouse. *Biol. Reprod.* **81**: 142–146.
81. Erdős, E. G. 1990. Angiotensin I converting enzyme and the changes in our concepts through the years. Lewis K. Dahl memorial lecture. *Hypertension*. **16**: 363–370.
82. Kregel, J. H., S. W. John, L. L. Langenbach, J. B. Hodgins, J. R. Hagaman, E. S. Bachman, J. C. Jennette, D. A. O'Brien, and O. Smithies. 1995. Male-female differences in fertility and blood pressure in ACE-deficient mice. *Nature*. **375**: 146–148.
83. Hagaman, J. R., J. S. Moyer, E. S. Bachman, M. Sibony, P. L. Magyar, J. E. Welch, O. Smithies, J. H. Kregel, and D. A. O'Brien. 1998. Angiotensin-converting enzyme and male fertility. *Proc. Natl. Acad. Sci. USA*. **95**: 2552–2557.
84. Yamaguchi, R., K. Yamagata, M. Ikawa, S. B. Moss, and M. Okabe. 2006. Aberrant distribution of ADAM3 in sperm from both angiotensin-converting enzyme (Ace)- and calyegin (Clgn)-deficient mice. *Biol. Reprod.* **75**: 760–766.
85. Leisle, L., E. T. Parkin, A. J. Turner, and N. M. Hooper. 2005. Angiotensin-converting enzyme as a GPIase: a critical reevaluation. *Nat. Med.* **11**: 1139–1140.
86. Fuchs, S., K. Frenzel, C. Hubert, R. Lyng, L. Muller, A. Michaud, H. D. Xiao, J. W. Adams, M. R. Capecchi, P. Corvol, et al. 2005. Male fertility is dependent on dipeptidase activity of testis ACE. *Nat. Med.* **11**: 1140–1142.
87. Yoshitake, H., H. Tsukamoto, M. Maruyama-Fukushima, K. Takamori, H. Ogawa, and Y. Araki. 2008. TEX101, a germ cell-marker glycoprotein, is associated with lymphocyte antigen 6 complex locus k within the mouse testis. *Biochem. Biophys. Res. Commun.* **372**: 277–282.
88. Kurita, A., T. Takizawa, T. Takayama, K. Totsukawa, S. Matsubara, H. Shibahara, M. C. Orgebin-Crist, F. Sando, Y. Shinkai, and Y. Araki. 2001. Identification, cloning, and initial characterization of a novel mouse testicular germ cell-specific antigen. *Biol. Reprod.* **64**: 935–945.
89. Fujihara, Y., M. Okabe, and M. Ikawa. 2014. GPI-anchored protein complex, LY6K/TEX101, is required for sperm migration into the oviduct and male fertility in mice. *Biol. Reprod.* **90**: 60.
90. Lin, Y., K. Mahan, W. F. Lathrop, D. G. Myles, and P. Primakoff. 1994. A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. *J. Cell Biol.* **125**: 1157–1163.
91. Primakoff, P., W. Lathrop, L. Woolman, A. Cowan, and D. Myles. 1988. Fully effective contraception in male and female guinea pigs immunized with the sperm protein PH-20. *Nature*. **335**: 543–546.
92. Phelps, B. M., P. Primakoff, D. E. Koppel, M. G. Low, and D. G. Myles. 1988. Restricted lateral diffusion of PH-20, a PI-anchored sperm membrane protein. *Science*. **240**: 1780–1782.
93. Baba, D., S. Kashiwabara, A. Honda, K. Yamagata, Q. Wu, M. Ikawa, M. Okabe, and T. Baba. 2002. Mouse sperm lacking cell surface hyaluronidase PH-20 can pass through the layer of cumulus cells and fertilize the egg. *J. Biol. Chem.* **277**: 30310–30314.
94. Honda, A., K. Yamagata, S. Sugiura, K. Watanabe, and T. Baba. 2002. A mouse serine protease TESP5 is selectively included into lipid rafts of sperm membrane presumably as a glycosylphosphatidylinositol-anchored protein. *J. Biol. Chem.* **277**: 16976–16984.
95. Yamashita, M., A. Honda, A. Ogura, S. Kashiwabara, K. Fukami, and T. Baba. 2008. Reduced fertility of mouse epididymal sperm lacking Prss21/Tesp5 is rescued by sperm exposure to uterine microenvironment. *Genes Cells*. **13**: 1001–1013.
96. Inoue, N., M. Ikawa, A. Isotani, and M. Okabe. 2005. The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. *Nature*. **434**: 234–238.
97. Miyado, K., G. Yamada, S. Yamada, H. Hasuwa, Y. Nakamura, F. Ryu, K. Suzuki, K. Kosai, K. Inoue, A. Ogura, et al. 2000. Requirement of CD9 on the egg plasma membrane for fertilization. *Science*. **287**: 321–324.
98. Le Naour, F., E. Rubinstein, C. Jasmin, M. Prenant, and C. Boucheix. 2000. Severely reduced female fertility in CD9-deficient mice. *Science*. **287**: 319–321.
99. Kaji, K., S. Oda, T. Shikano, T. Ohnuki, Y. Uematsu, J. Sakagami, N. Tada, S. Miyazaki, and A. Kudo. 2000. The gamete fusion process is defective in eggs of Cd9-deficient mice. *Nat. Genet.* **24**: 279–282.
100. Inoue, N., D. Hamada, H. Kamikubo, K. Hirata, M. Kataoka, M. Yamamoto, M. Ikawa, M. Okabe, and Y. Hagiwara. 2013. Molecular dissection of IZUMO1, a sperm protein essential for sperm-egg fusion. *Development*. **140**: 3221–3229.
101. Coonrod, S. A., S. Naaby-Hansen, J. Shetty, H. Shibahara, M. Chen, J. M. White, and J. C. Herr. 1999. Treatment of mouse oocytes with PI-PLC releases 70-kDa (pI 5) and 35- to 45-kDa (pI 5.5) protein clusters from the egg surface and inhibits sperm-oolemma binding and fusion. *Dev. Biol.* **207**: 334–349.
102. Kawagoe, K., D. Kitamura, M. Okabe, I. Taniuchi, M. Ikawa, T. Watanabe, T. Kinoshita, and J. Takeda. 1996. Glycosylphosphatidylinositol-anchor-deficient mice: implications for clonal dominance of mutant cells in paroxysmal nocturnal hemoglobinuria. *Blood*. **87**: 3600–3606.
103. Alfieri, J. A., A. D. Martin, J. Takeda, G. Kondoh, D. G. Myles, and P. Primakoff. 2003. Infertility in female mice with an oocyte-specific knockout of GPI-anchored proteins. *J. Cell Sci.* **116**: 2149–2155.
104. Bianchi, E., B. Doe, D. Goulding, and G. J. Wright. 2014. Juno is the egg Izumo receptor and is essential for mammalian fertilization. *Nature*. **508**: 483–487.
105. Mashiko, D., Y. Fujihara, Y. Satouh, H. Miyata, A. Isotani, and M. Ikawa. 2013. Generation of mutant mice by pronuclear injection of circular plasmid expressing Cas9 and single guided RNA. *Sci. Rep.* **3**: 3355.
106. Wang, H., H. Yang, C. S. Shivalila, M. M. Dawlaty, A. W. Cheng, F. Zhang, and R. Jaenisch. 2013. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*. **153**: 910–918.
107. Six, E., D. Ndiaye, Y. Laabi, C. Brou, N. Gupta-Rossi, A. Israel, and F. Logeat. 2003. The Notch ligand Delta1 is sequentially cleaved by an ADAM protease and gamma-secretase. *Proc. Natl. Acad. Sci. USA*. **100**: 7638–7643.
108. Satouh, Y., N. Inoue, M. Ikawa, and M. Okabe. 2012. Visualization of the moment of mouse sperm-egg fusion and dynamic localization of IZUMO1. *J. Cell Sci.* **125**: 4985–4990.
109. Miyado, K., K. Yoshida, K. Yamagata, K. Sakakibara, M. Okabe, X. Wang, K. Miyamoto, H. Akutsu, T. Kondo, Y. Takahashi, et al. 2008. The fusing ability of sperm is bestowed by CD9-containing vesicles released from eggs in mice. *Proc. Natl. Acad. Sci. USA*. **105**: 12921–12926.